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A NEW THEORY ON THE MECHANICS OF CILIARY AND FLAGELLAR MOTILITY. I. SUPPORTING OBSERVATIONS [1]

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Over the course of several years, observations on negatively stained and living cilia and sperm flagella from a number of different sources have been accumulated in this laboratory. Collectively, these findings have provided growing evidence in favor of a new and relatively simple explanation of the mechanics of motility in such organelles, which was outlined in a very preliminary form earlier (Costello and Henley, 1970). In the present paper, the supporting data will be examined; the new theory, and the relation of these data to it are outlined in a second communication (Costello, 1973).

In general, the evidence indicates that under certain conditions, doublet microtubules bend into coils or helical configurations, while at the same time the singlets straighten, or stiffen and remain straight for a finite period. I believe that these phenomena are directly related to the mechanism of ciliary and flagellar motility.

Materials and Methods

The biological material consisted of a number of invertebrates, principally freshwater and marine flatworms. The freshwater material was collected from streams in the vicinity of Chapel Hill, North Carolina, and from Stone Mountain, Georgia (*Mesostoma georgianum*). The marine material was furnished by the Supply Department of the Marine Biological Laboratory at Woods Hole, Massachusetts, or collected at Pacific Grove, California (*Polychoerus carmelensis*). A great deal of this material was prepared for another purpose and studied largely by Dr. Catherine Henley, to whom I am greatly indebted, both for the opportunity of examining her extensive collection of electron micrographs and for the use of a number of these for measurements and to illustrate this paper.

The material examined included the following: the marine acoels *Polychoerus carmelensis, P. caudatus, Childia spinosa* (*groenlandica?*) and *Anaperus gardineri;* the freshwater rhabdocoels *Mesostoma georgianum, Microdalyellia* sp. and *Macrostomum* sp.; the marine alloeocoels *Monoophorum* sp., *Monocelis* sp. and *Plagiostomum* sp.; the triclads *Dugesia tigrinum* (freshwater) and *Bdelloura candida* (marine); the marine polyclads *Stylochus zebra* and *Notoplana* sp.; the freshwater rhynchocoel *Prostoma rubrum;* the marine polychaete annelid *Chaetopterus pergamentaceus;* the earthworm *Lumbricus terrestris;* the prosobranch freshwater mollusc *Goniobasis proxima;* and several unidentified polyclads collected off the North Carolina coast.

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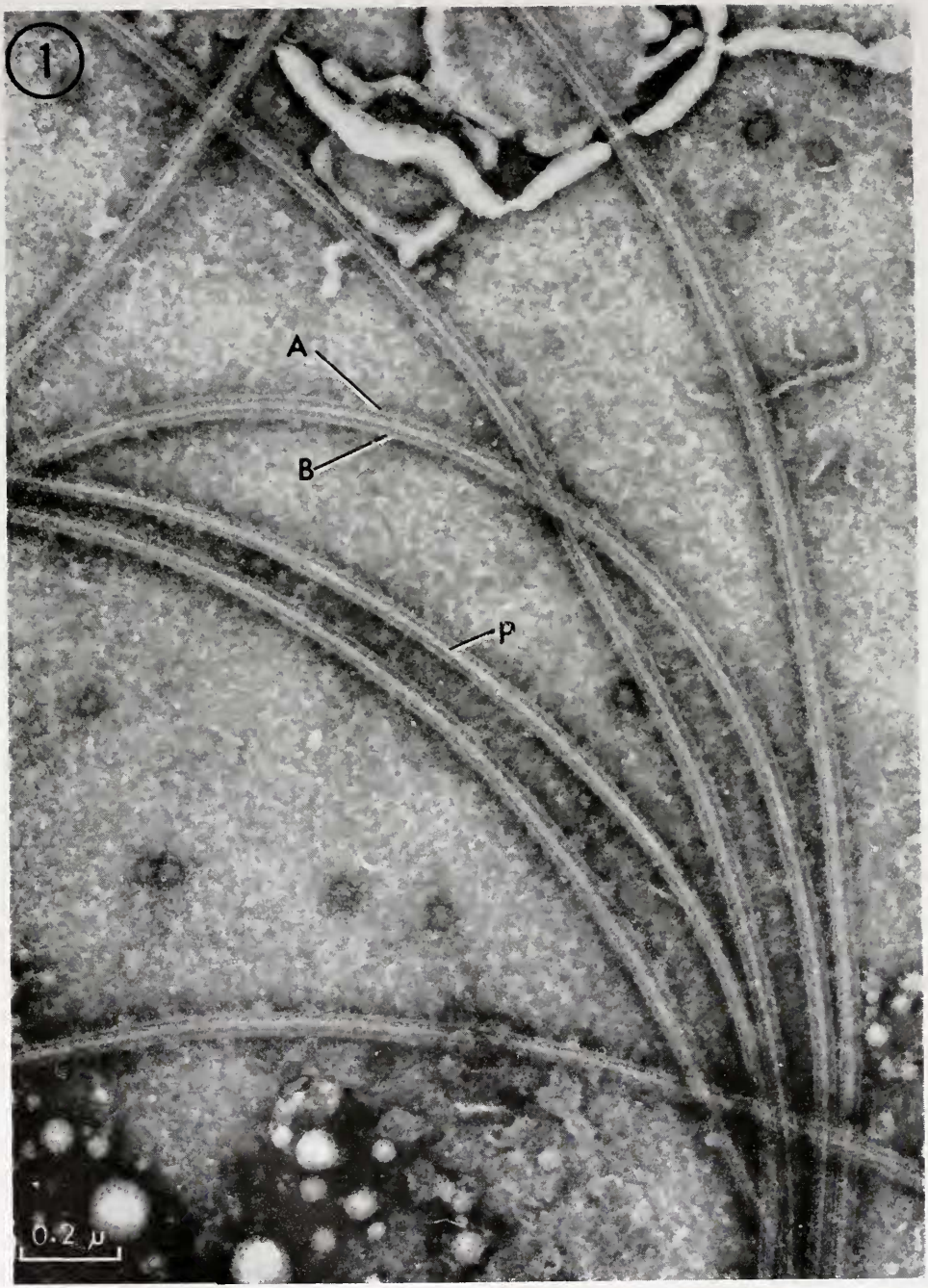


FIGURE 1. Part of a group of doublet microtubules of an isolated cilium of *Mesostoma*, showing relation of bending to the A- and B-subtubules (A, B) and partition wall (P) between them. See text; 70,000 ×.

The phosphotungstate negative staining (1% PTA, pH 6.8) of the spermatozoa, of earlier stages of spermiogenesis, and of the surface cilia was supplemented by study of material fixed, sectioned and stained for electron microscopy, and by very extensive studies by ordinary light and phase contrast microscopy of living and fixed cilia and spermatozoa, both normal and PTA-treated. For details of techniques, see Costello, Henley and Ault (1969), Henley, Costello, Thomas and Newton (1969), Henley (1970a) *etc.* An extensive pretreatment of earthworm spermatozoa with distilled water, prior to PTA maceration and negative staining, was used in demonstrating that the singlets are attached to each other (Henley, 1970b) and show exceptionally rigid elastic properties; see Henley (1973) for details.

All micrographs were made with the Zeiss 9A or 9S2 electron microscope.

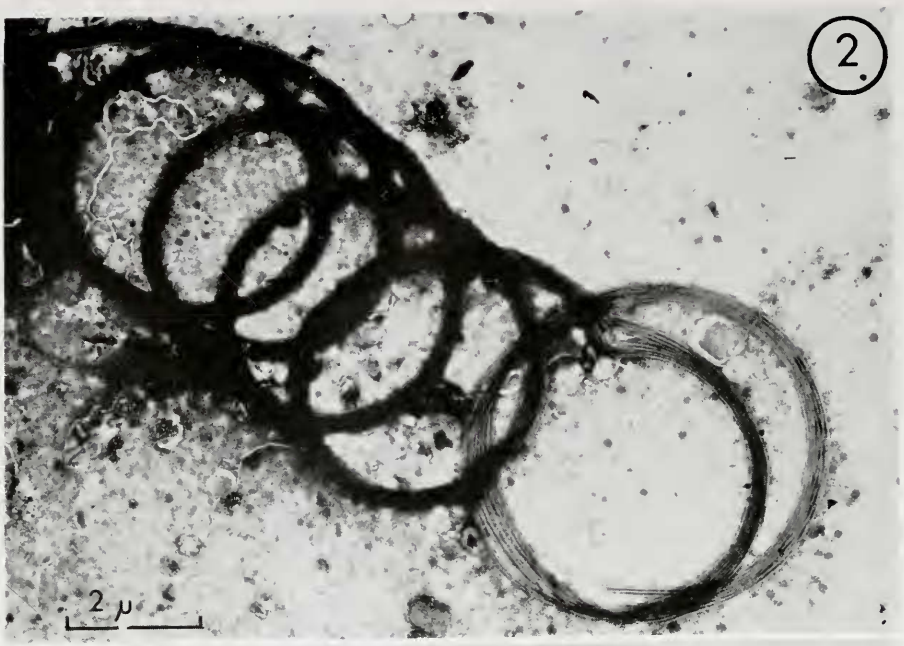
Observations

Evidence for coiling and straightening

Evidence that induced coiling of the doublets is of importance in the interpretation of the bending process in ciliary and flagellar motility is supplied by observation of isolated groups of axonemes (Figs. 1–6; see also Henley *et al.*, 1969), and analysis of selected electron micrographs, such as Figures 7 and 8.

From the arrangement of the coils of the doublets, in relation to the partition wall between subtubules A and B, and from the location, on the coils, of the partially macerated spokes, it is concluded that the bending of the doublet occurs in a direction at right angles to the partition wall, and away from the region where the dynein arms were attached (see Fig. 1 in the following paper). That is, the flexible, tree-like remnants of the spokes, which are attached at regular intervals to the A-subtubule, extend out on the convex side of the flattened doublet coils (Fig. 6 and, more strikingly, in Henley *et al.*, 1969, Fig. 4, page 853). The partition wall is most often perpendicular to the substratum and one side of both the A- and the B-subtubule is in contact with the grid coating (Fig. 1). In addition, the A-subtubule, which forms a complete circle, shows up as wider than the B-subtubule, which is incomplete. So far as I can ascertain from the literature, investigators considering modes of axonemal motility have not realized that the doublets must do their active bending in a particular plane in relation to their doublet structure. It is a matter of simple logic to assume that the mode of active bending of doublets is dependent upon their ultrastructural organization, including the location of the ATPase, and that because of this organization, they cannot bend equally easily in all planes. If one is thinking in terms of a sliding filament theory as the basis of movement (Satir, 1965, 1968; Summers and Gibbons, 1971; Sleigh, 1973), in axonemal motility there must be a bending of the filaments accompanying the sliding of filaments upon one another (see also Brokaw, 1971, 1972). Mere sliding of straight, unhampered microtubules upon each other could produce only a jack-in-the-box effect at their tips. Horridge (1965) provided evidence for both sliding and bending of doublets in the macrocilia of *Beroë*, but it was uncertain at that time how this might apply to single axonemes.

Negatively stained preparations made by macerating cilia to the point where the membrane and matrix have been removed but with the doublets still attached to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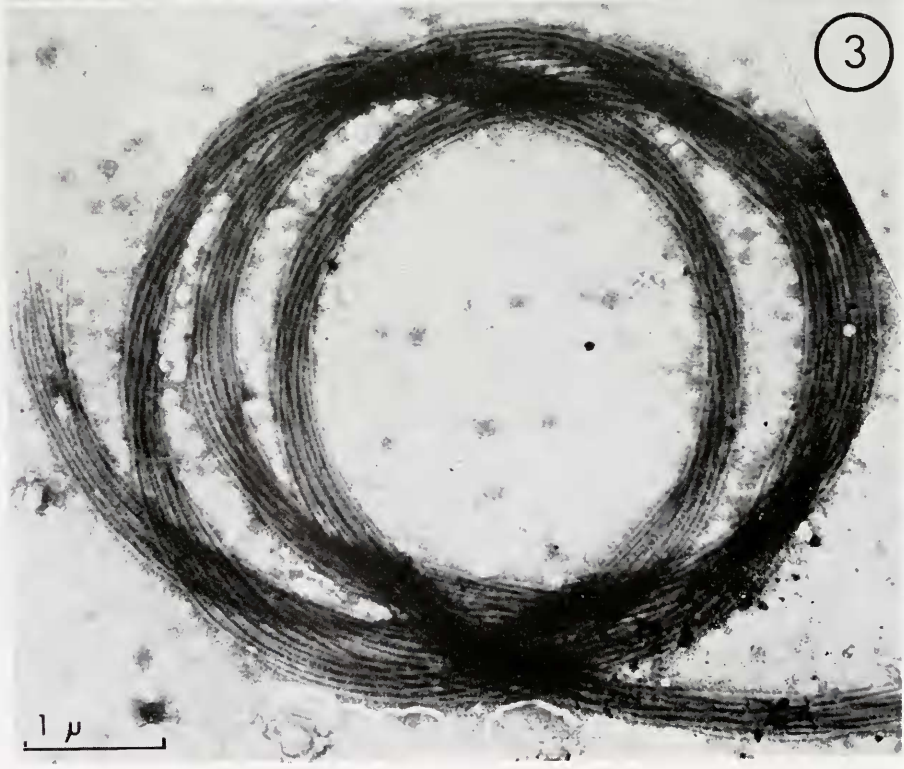
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the basal plate show the doublets flared out in 9 arcs which do not close to form complete circles (Figs. 7, 8). Note that in each of these cases, four of the doublets bend to one side, and five in the opposite direction. The central singlets in Figure 7 are in the stiffened condition and in Figure 8 they are in the relaxed condition. After an appropriate amount of maceration, such configurations are common. The overall picture is much like the diagram of the form or positions of a cilium during various effective and recovery portions of the stroke. The lengths measured along these ciliary doublets for a large number of turbellarians were 12 to 15 microns. On the other hand, axonemal isolates of long flagella macerated to a somewhat lesser degree show repeated coiling into circles of quite uniform diameter, usually averaging from 3 to 4.5 microns (see Figs. 2 and 3). The circumference of a single coil of a flagellar doublet thus corresponds approximately to the length of a doublet of a ciliary axoneme. On the basis of these comparisons, a complete arc or a single coil would correspond to the length of axoneme involved in $\frac{1}{2}$ wavelength, two coils to that of a whole wavelength.

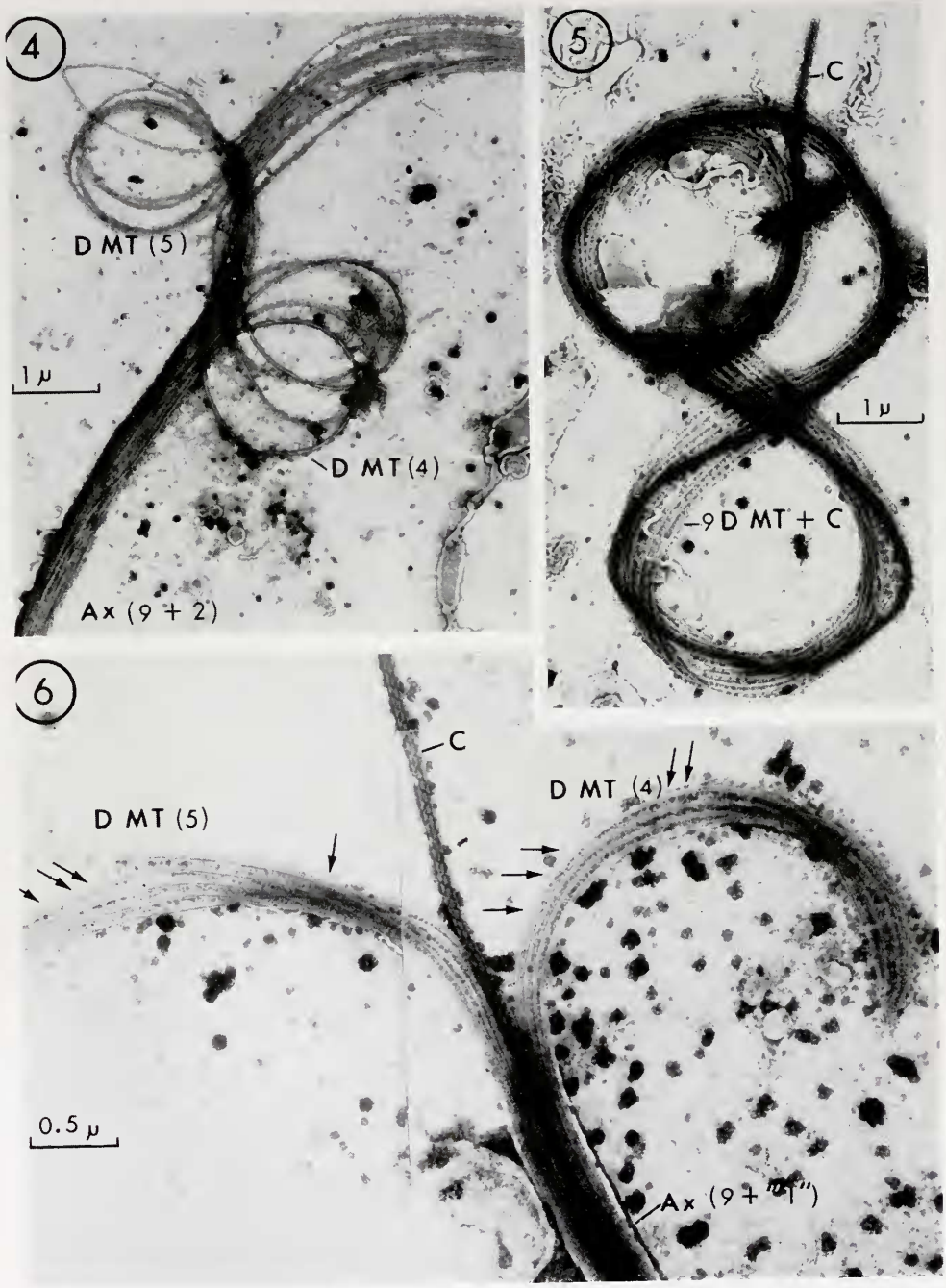
In "9 + 1" flagella, if maceration with the phosphotungstate is of insufficient duration to free the central core, this structure remains encompassed by the doublets and bends as the axoneme coils. Under these circumstances (which involve, also, incomplete matrix removal) the coils may be of varying diameters. With a greater degree of maceration, all or part of the central core "escapes" from the doublets (Figs. 5 and 6) and is very frequently found extending along a relatively straight course for considerable distances—as much as 73 microns in one case (Henley *et al.*, 1969).

A number of interesting configurations have been observed after PTA treatment of various spermatozoa. For example, Figure 4 depicts twin looping in a partially macerated axoneme of a spermatozoon of *Chaetopterus*. The portion of the axoneme (Ax) nearer the basal plate is less macerated than the more distal portion, and the looping has occurred at the junction of the two regions. That the "clockwise" (4 doublets) and "counterclockwise" (5 doublets) loops are related to the two lateral half doublet groups (with doublets #5 and #6 presumably in the latter) is a reasonable certainty.

Occasionally there are 6 doublets bending to one side and only three to the other in both the isolated macerated cilia and flagellar tips. In these cases the six were often seen to consist of one group of four and another group of two doublets, with the latter identifiable as the #5–6 pair. The more usual pattern of four doublets bending to one side while five doublets bend to the other side is thus logically modified.

Figure 5 is an electron micrograph of a portion of a "9 + 1" axoneme of a PTA-treated spermatozoon of *Dugesia*. The core is included within the coils until it becomes free. At this point the doublets continue their bent course and the fragment of escaped core is straight. The significance of the figure-of-eight, with "clockwise" and "counterclockwise" bends of the axoneme, is probably related to the two lateral half-groups of doublets bending in opposite directions. Figure 6 is a similarly treated portion of a spermatozoan axoneme of *Dugesia*, at the point

FIGURES 2 and 3. PTA-treated sperm axonemes of *Goniobasis*, showing induced coiling. See text; 8900 × and 18,600 ×, respectively.



FIGURES 4, 5 and 6. Partially macerated PTA-treated 9 + 2 sperm axoneme of *Chaetopterus* (Fig. 4) and "9 + 1" sperm axonemes of *Dugesia* (Figs. 5 and 6). See text; Figure 4,

of escape of the straight core from the doublets. There are 4 doublets bending "clockwise" and 5 doublets bending "counterclockwise." All have remnants of spokes visible on their *convex* surfaces.

Identification of doublets by number

In certain electron micrographs of ciliary isolates that had been optimally macerated and negatively stained, the #5–6 attached doublet pair could be identified with considerable certainty. In addition, the points of insertion of all the doublets into the basal plate could sometimes be seen. Enlargement of these micrographs and careful examination made possible identification of the doublet microtubules by number. Figure 8 is the best example of this, and all doublets could be identified in Figure 7, also.

Figure 8 shows a cilium of *Mesostoma georgianum*, with the #5–6 doublets demonstrably attached to one another over part of the basal two-thirds of their lengths, and flared apart in opposite directions distally. This suggests that, in this form at least, the dynein arms-back bridge connectives are quite stable, and not totally disrupted or macerated by the PTA treatment. Since the #5–6 doublets were readily identifiable, it was possible to identify also, by number, every other individual doublet, and to correlate their identities with the direction of their bending. Thus far, from this and other examples, the data are consistent with the view that in cilia the doublets of one lateral half (#2, #3, and #4) bend in a direction opposite to that of the doublets of the other lateral half (#7, #8 and #9). With little maceration and with the matrix intact the axoneme bends as one unit.

Induced coiling of glutaraldehyde-fixed spermatozoa

Additional evidence that PTA induces sperm flagella to become helically coiled has been obtained. Spermatozoa of the marine annelid *Chaetopterus* were taken from the parapodia of a male and fixed in a cacodylate-buffered glutaraldehyde fixative devised for acoel flatworms. The spermatozoa did not coagulate into a mass, but were fixed, in suspension, essentially in a straight form. Diluting the glutaraldehyde-fixed spermatozoa with buffer-sucrose wash did not alter this form (Fig. 9). Diluting the glutaraldehyde-fixed spermatozoa 1:4 with 1% PTA at pH 6.8, however, induced the straight tails to assume strikingly helical patterns. Observation by phase contrast microscopy revealed that the tails of the intact fixed spermatozoa became curved, looped, and then helical, with up to three gyres, in the course of 20 to 30 minutes (Fig. 10). Four per cent PTA at pH 2, mercuric chloride and lead nitrate solutions also induced excellent spiralling of the tails. It is of interest that the form of the "wave" was clearly not planar. The changes occurred so slowly in these dead, fixed spermatozoa that actual movement of the flagellum was not perceptible. While I have not been able to reverse the coiling with specific agents, the helices are not rigid. The tails straighten out readily when a directed flow of the surrounding medium is induced under the coverglass.

11,665 ×; Figure 5, 11,665 ×; Figure 6, 23,750 ×; Ax, axoneme; C, core; D MT, doublet microtubules.

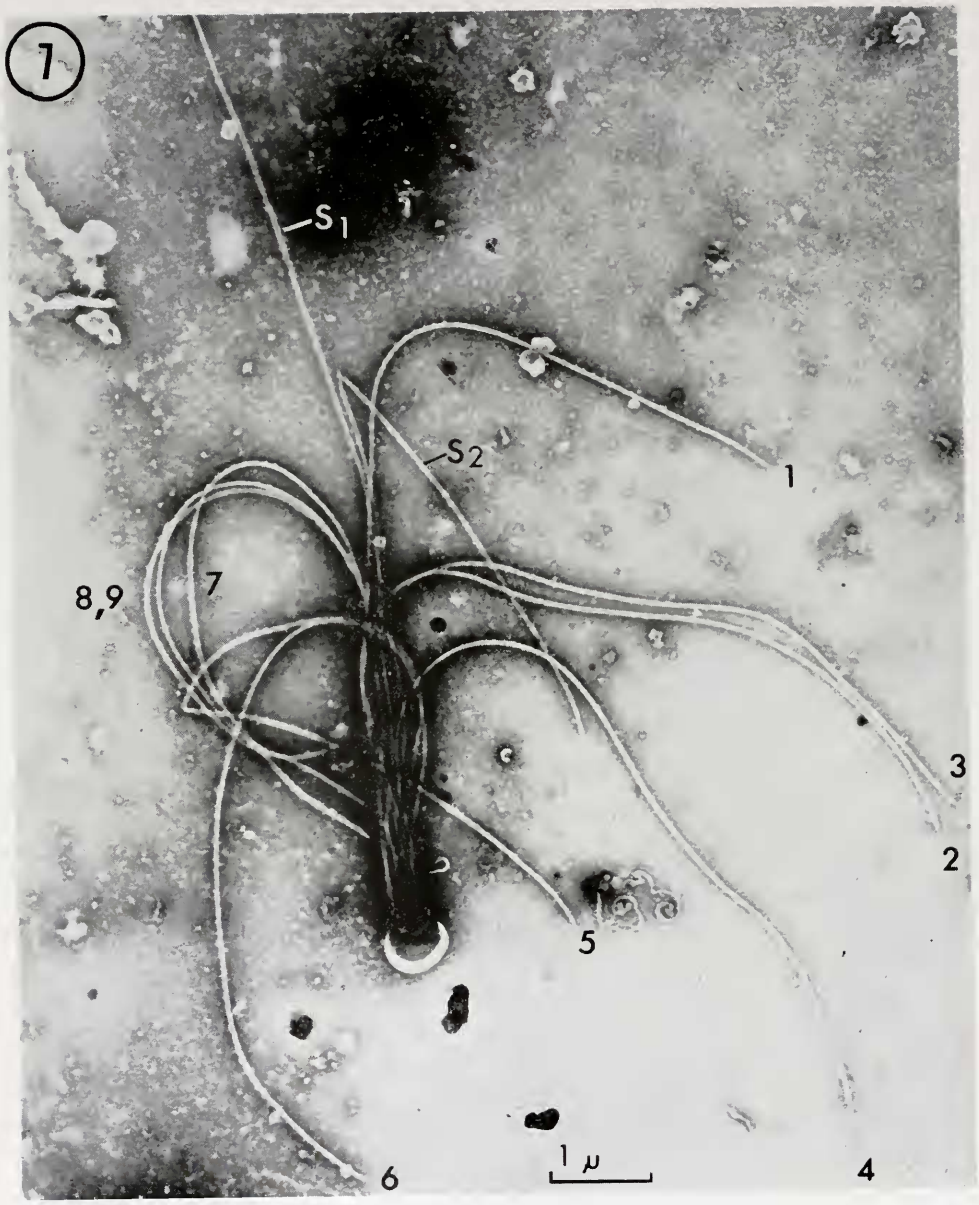


FIGURE 7. Macerated, negatively stained (by PTA) surface cilium of *Mesostoma*, with bent doublets (identified by number) and straight singlets (S_1, S_2). Doublets #1–4 bend to right, #5–9 to left; doublets #5 and #6 attached over part of length; 13,250 ×.

These results have been reported, thus far, only in abstract (Costello and Henley, 1969).

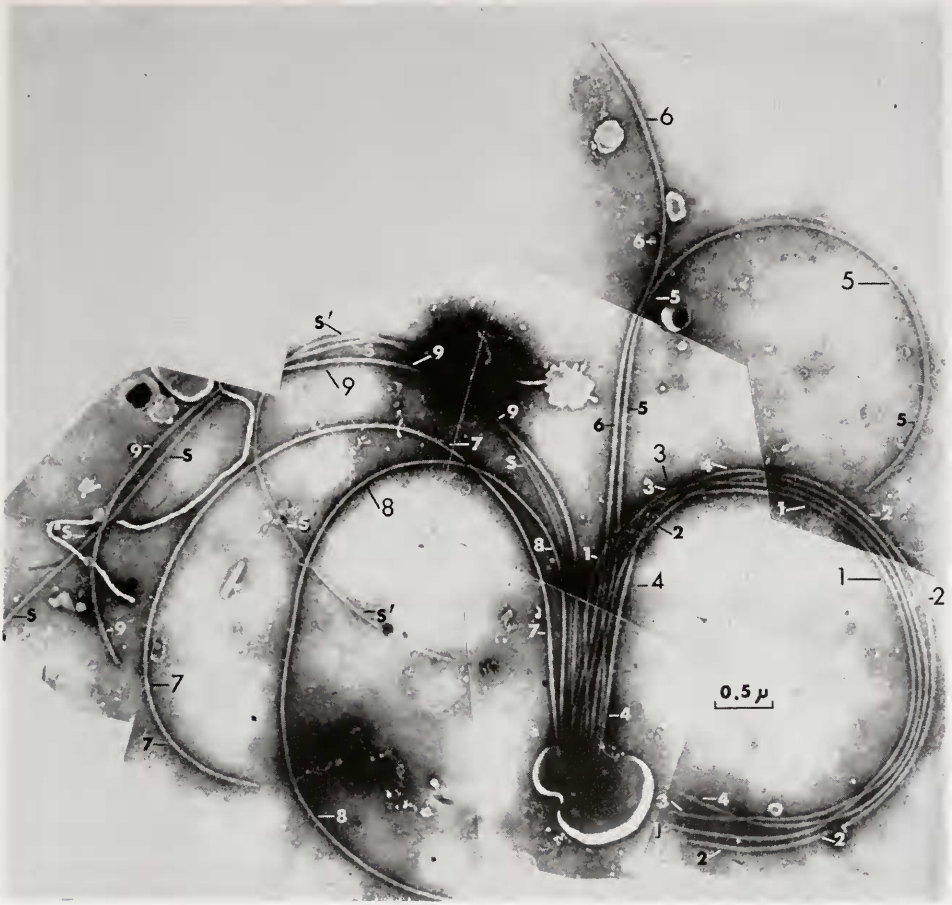


FIGURE 8. Macerated negatively stained (by PTA) surface cilium of *Mesostoma* with clearly identifiable doublets and singlets S and S'. Doublets #1–5 bend to right, and #6–9 to left. Each doublet is identified by a large number, plus smaller numbers to trace its course; 15,390 ×.

Rigidity of central singlet microtubules in earthworm sperm

The spermatozoon of the earthworm has what superficially appears to be the conventional 9 + 2 pattern of microtubules. However, the two central singlets can be seen, in both sectioned and negatively stained material, to be connected to one another by short bridges at regular intervals, and to be accompanied by longitudinal paired fibrous elements which are clearly solid and smaller in diameter than microtubules (Henley, 1973). Under certain conditions of negative staining this complex of cross-linked singlets plus paired fibrous elements is found to fall in configurations similar to those seen in negatively stained "9 + 1" cores. The evidence suggests that the central complex in earthworm spermatozoa has elastic properties very different from those exhibited by the doublets, and that this com-

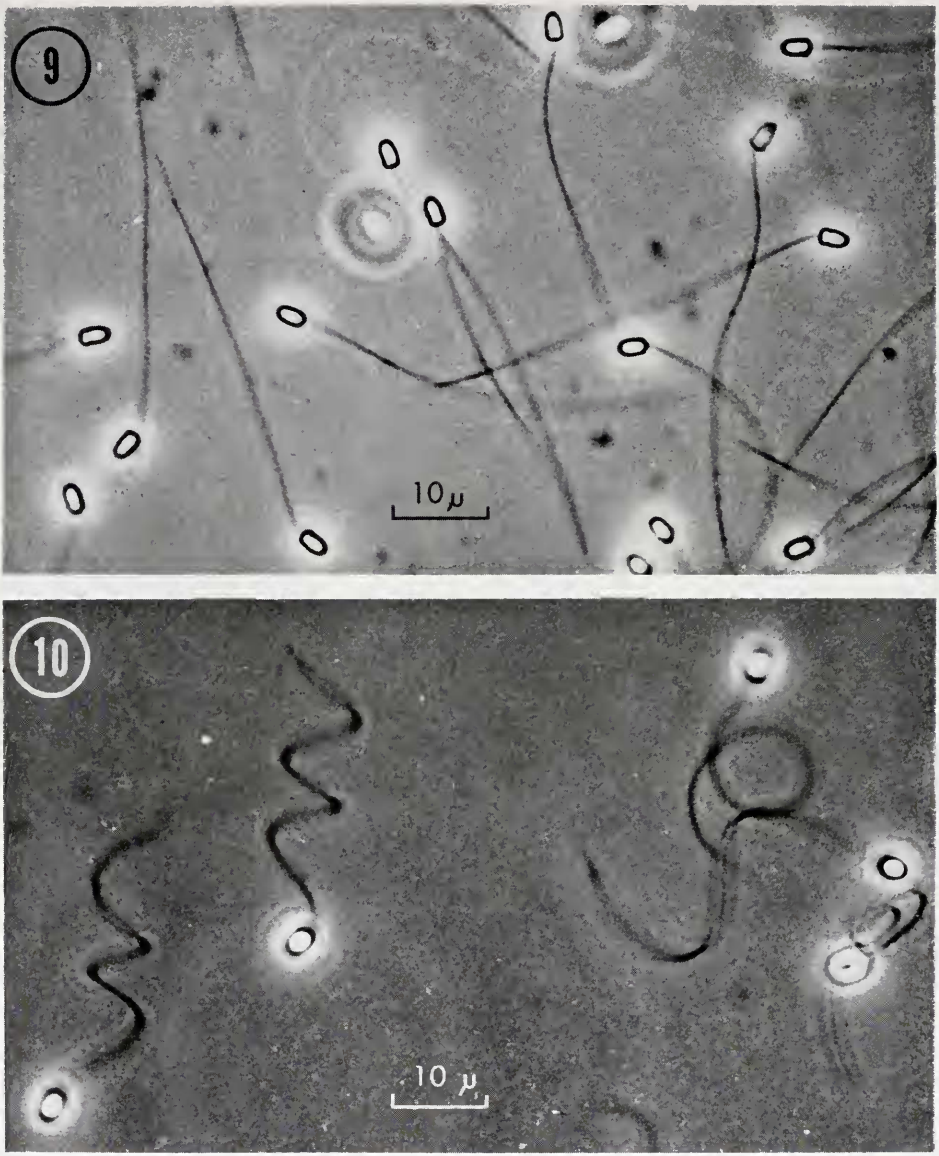


FIGURE 9. *Chaetopterus* spermatozoa fixed in cacodylate-buffered glutaraldehyde. Suspension was later diluted with buffer wash. Note essentially straight tails; phase-contrast, 1280 ×.

FIGURE 10. *Chaetopterus* spermatozoa fixed in cacodylate-buffered glutaraldehyde, and later diluted with 1% PTA, pH 6.8. Note helically coiled tails; phase-contrast, 1280 ×.

plex is clearly involved in a stiffening function. The connectives could inhibit or prevent the sliding of singlets, relative to each other, that would be expected to

accompany lateral bending. Gibbons (1961) found sometimes single, sometimes double bridges between the central singlets of lamellibranch cilia.

During the course of the present investigation we have found clear-cut evidence for regularly repeated connectives between the central singlets of the cilia of *Hydrolimax grisea*, also.

Helical-protofibrillar transitions

Another possibly pertinent series of observations relates to the transitions between the protofibrillar arrangement and a helical configuration of subunits in the microtubules. Such transitions were observed by Henley in negatively stained *Mesostoma* sperm in 1969, and described in detail by Thomas (1970) for PTA-treated *Stylochus* sperm and by Thomas and Henley (1971) for spermatozoa of *Macrostomum*. Transitions were found both in cortical singlet and in doublet microtubules. The singlets, which characteristically have a helical arrangement of the subunits, often showed a transition to the protofibrillar condition. Thomas (1970, page 231), states that neither the protofibrillar nor the helical configuration appears to be characteristic of the intact unmacerated doublets, but that both conditions were to be found in her PTA-treated material. However, extended observations on a number of different species of spermatozoa make it increasingly clear that the protofibrillar condition is characteristic of the straight, intact, PTA-treated doublet. But, transitions do occur.

If subtubule B (the incomplete subtubule) is macerated away, leaving subtubule A (the complete member), then the subunits of A assume a helical arrangement (Thomas, 1970, Figs. 12–15). Similar transitions are found in subtubule A of earthworm axonemal doublets when the B member is macerated away (see Fig. 9 in Henley, 1973).

The evidence suggests that singlets and doublets, in their "normal" states, exist in opposite phase—that is, singlets with subunits arranged in the helical state and doublets with subunits in the protofibrillar state.

Cohen, Harrison and Stephens (1971) have obtained x-ray diffraction patterns of wet gels of A-subtubules from sea urchin sperm-tail doublets. The results indicate that subunits with a 40–50 Å packing diameter form filaments, alternately half-staggered, parallel to the tubule's axis. A 12- or 13-stranded structure best fits the x-ray diagram. A dried A-subtubule sample gives an x-ray diffraction pattern with a strong meridional reflection at 40 Å, rather than the off-meridional diffraction seen with native specimens. The lateral bonds of the surface lattice of the A-subtubule are destroyed by drying, leaving the stronger axial bonds connecting simple linear arrays of subunits (see, also, Stephens, 1973).

Bearing in mind the fact that A-subtubules may differ from intact doublets (as discussed above), the evidence is compatible with the idea that a conformational change in subunit arrangement may be involved in microtubule bending. The possible implications of all these observations will be considered in the next paper, in relation to a theory which attempts to account for the mechanics of ciliary and flagellar motility.

Summary

1. Controlled partial maceration and negative staining of ciliary and flagellar axonemes (with phosphotungstate) have revealed that under these conditions: (a) Doublet microtubules have an inherent tendency for bending or coiling; (b) Central singlet microtubules of 9 + 2 axonemes, or the cores of the "9 + 1" flagellar axonemes, stiffen or straighten; (c) Active bending of the doublets occurs in the direction away from their dynein arms, so that the A-subtubule is on the convex side.

2. In partially macerated 9 + 2 axonemal isolates, examination of electron micrographs indicates that the bending of the doublets of one lateral half is in a direction opposite to that of the doublets of the other lateral half. This is a consequence of (1, c) above.

3. Evidence is presented for (2) above, in micrographs showing the direction of bending of specifically identified doublet microtubules of isolated ciliary axonemes.

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